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14. ABSTRACT

Background: Prostate cancer (PCa), a leading cause of male mortality, development and progression is dependent upon androgen and androgen receptor (AR) signaling. Current therapies target androgen production and/or AR signaling. Evidence suggests that AR in some tumors may escape therapy through mechanisms that likely involve splicing. Purpose: To better understand splicing during PCa development or progression. Scope: Observations suggest that the splicing factor (SF2) may contribute to PCa. Findings: i) IHC analysis, suggests SF2 is elevated in human PCa specimens, ii) PCa-derived cell model systems with altered levels of SF2 that mimic human disease, suggests proliferative phenotype, iii) Knockdown of SF2, suggests PCa-specific splicing. Recent progress: i) Immunoblot analysis of SF2 in primary PCa tissue lysates validated IHC findings, ii) Knockdown of SF2 promotes G2/M arrest, iii) Tissue recombination assays yielded no identifiable glandular structures, iv) An ex vivo primary PCa culture system was developed to evaluate future splicing and therapeutic potential.

15. SUBJECT TERMS
SF2, cyclin, androgen receptor

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INTRODUCTION

Background/Subject: Prostate cancer (PCa) is one of the most prevalent non-cutaneous cancers in men and the second leading cause of mortality after lung cancer (1). PCa is a multi-factorial disease with genetic, hormonal, and environmental components. A critical facet of PCa development and progression is its dependence on androgen signaling as mediated by the androgen receptor (AR) (2). Current therapeutic options range from surgery/radiation for localized disease to deprivation of androgen and/or AR signaling in more aggressive, advanced/metastatic disease. Chemotherapy is not particularly beneficial; however, androgen deprivation therapies (ADT) result in effective disease management for 2-3 years before incurable Castrate-resistant PCa (CRPC) develops (3).

General Purpose: To better understand splicing in PCa and identify novel therapeutic pathways. Especially, as recent evidence suggests isoforms of AR, potentially derived through alternative splicing, may contribute significantly to disease progression (4). Unfortunately, little is known concerning the mechanisms, pathways, and/or components of splicing in PCa.

Overall Scope: Previously it been demonstrated that cyclin D1, a key modulator of androgen/AR-dependent transcription and proliferation, is alternatively spliced in PCa (5). Published data have suggested that SF2 and cyclin D1 isoforms have oncogenic functions (6, 7) suggesting a potential link between splicing and proliferation. Based on preliminary data (now published (8)), the current proposal is geared towards determining the consequence of SF2 function and splicing in the context of PCa.

BODY

The current findings are summarized within the overall tasks, aims, and subaims (specific details and projected timelines were provided in the initial Proposal and Statement of Work, respectively) and are indicated in the Current Accomplishments sections. Relevant data that is published and directly pertains to the current Tasks will be summarized and cited or will be provided in reproduction, as indicated, for ease of committee evaluation. Previously reported progress is summarized under the Completed Accomplishments sections. Those studies from previous updates that are essential for the writing of an RO1 (as described in the reportable outcomes section) are indicated and summarized under the Recent Accomplishments sections. Subaims that are to be addressed in subsequent years are indicated and relevant data that is applicable to multiple subaims will also be indicated.

TASK 1

Overview: The overall goal of Task 1 is to essentially determine the impact (Aim A) and relevance (Aim B) of SF2 function and splicing in the context of PCa.

Aim A: is focused on the identification of PCa cell model systems and their subsequent manipulation of SF2 to mimic human disease (Subaim 1). The intended goal is to characterize the pathways and tumorigenic activity of these modified cell lines *in vitro* (Subaim 2) and eventually determine specificity through RNAi technology and inhibitory treatments that alter functional activity (Subaim 3 - to be addressed in subsequent years).

Subaim 1: Generate PCa cells to mimic human disease.

Summary: Preliminary data, in the initial Proposal, from a publically available gene expression database suggests that mRNA for SF2 is elevated in PCa.

Strategy: Identify cell model systems that are representative of PCa and are amenable to manipulation of SF2 in order to mimic disease.

Completed Accomplishments (From 1st Annual Update):
Critical to success of the proposal; it was determined, by immunoblot analysis, that among the well-characterized hormone-dependent PCa cell model systems available, LNCaP cells expressed the lowest levels of SF2 and would be the best model system to mimic elevated SF2 as suggested by the preliminary data. Furthermore, it was determined that VCaP cells express the most SF2 and would be best suited for knockdown studies of SF2.

Recent Accomplishments (From 2nd Annual Update):

Generation of a stable LNCaP cell line that expresses a nuclear T7-tagged SF2

Previously, a T7-tagged SF2 construct was used to generate a lentivirus for efficient transduction of normal and PCa cells. Using this construct, LNCaP cells were infected with T7-SF2 or Luciferase control and stable cell lines were selected for using puromycin. As shown by immunoblot analysis in **Figure 1**, specific induction of T7-SF2 was observed in LNCaP cells. Furthermore, T7-SF2 was localized to the nucleus as determined by immunofluorescence and is consistent with previously published literature using the T7-tagged SF2.

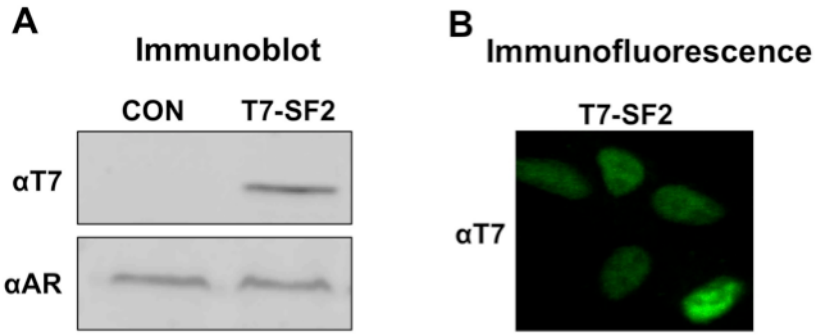

Figure 1. Stable LNCaP cells expressing nuclear T7-SF2.

A) Representative immunoblot for the T7-epitope (upper) and endogenous androgen receptor (AR) used as a loading control (lower) in stable LNCaP cells transduced with: lentiviral control (CON) or T7-tagged SF2 (T7-SF2). *Note*: AR is used as a loading control to demonstrate that elevated T7-SF2 does not alter AR levels.

B) Representative confocal image from immunofluorescence studies for the T7-epitope (FITC label, Green) in stable LNCaP cells transduced with T7-SF2. *Note*: T7-SF2 was co-localized with DAPI (data not shown) to the nucleus and immunostaining for T7 was not detectable in CON LNCaP cells (data not shown).

Subaim 2: Characterize tumorigenic activity of PCa cell lines *in vitro*.

Summary: The capacity and/or pathways of SF2 that promote tumor progression are unknown in PCa. While preliminary evidence indicates that SF2 mediates alternative splicing of cyclin D1, it does not rule out the possibility that other pathways may also contribute. Similarly, as D-cyclins are known to modulate androgen/AR-dependent transcription in PCa cells it is feasible that potential feed-forward and –backward transcriptional mechanisms are invoked that may influence SF2-mediated functions.

Strategy: Develop over-expression systems in LNCaP cells to study the contributions on gene expression and/or splicing as well as study the tumorigenic potential *in vitro*. Furthermore, establish a stable cell line for future *in vivo* studies outlined in Task 2.

Completed Accomplishments (From 1st Annual Update):

Transient over-expression of D-cyclin in LNCaP cells was performed to evaluate the overall transcriptional impact on AR-dependent signaling and these data were recently published (9). Preliminary evidence from this study identified gene expression changes in *CLK1*. Importantly, *CLK1* is a known kinase that serves as a critical signaling node to regulate the functional activity of SF2. While preliminary, this observation suggests a novel regulatory loop between D-cyclins and SF2. Moreover, robust and transient SF2 over-expression verified altered D-cyclin splicing [consistent with recently published data (8)] and the transcription of known SF2-associated genes (*e.g.*, *CLK1* and *C1qBP*) while having minimal impact on genes not known to be associated with SF2 (*e.g.*, *KHDRBS3*). These data will provide valuable information related to dissecting signaling pathways as part of the future studies.

Recent Accomplishments (From 2nd Annual Update):

Stable T7-SF2 expression in LNCaP cells promotes cellular proliferation and growth

These data were the central focus of an RO1 proposal (described below in the reportable outcomes section) designed to identify the mechanism behind SF2-mediated proliferation and growth. As shown in **Figure 2**, proliferation analysis using bromodeoxyuridine (BrdU) incorporation indicated that LNCaP cells expressing T7-SF2 have an approximate 1.6-fold increase in the number of cells in S-phase. Moreover, using growth curve analysis, LNCaP cells expressing T7-SF2 have increased cell numbers by day 4. Combined these data indicate that elevated SF2 promotes proliferation and growth in LNCaP cells.

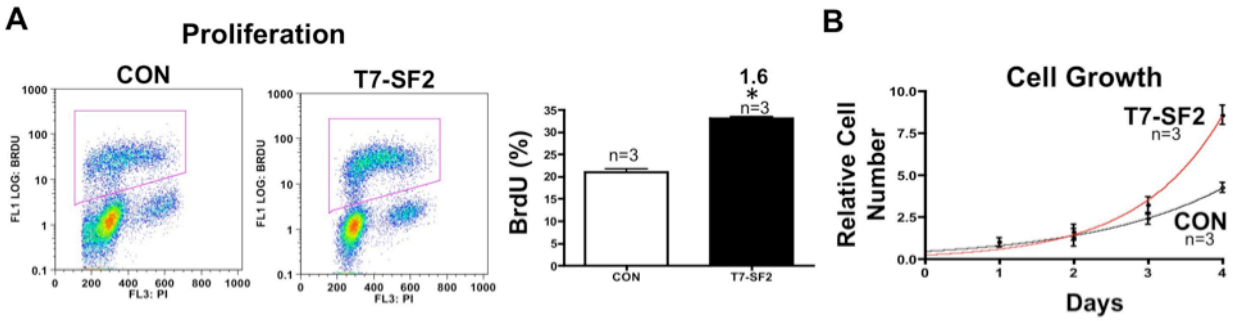

Figure 2. T7-SF2 increases proliferation and cell growth in LNCaP cells.

A) ***Left Panel***, Representative density plots of bivariate flow cytometry from stable LNCaP cells transduced with: lentiviral control (CON) or T7-tagged SF2 (T7-SF2). The x-axis denotes cells labeled with propidium iodide (PI), indicating DNA content. The y-axis denotes cells labeled for 2hr with bromodeoxyuridine (BrdU), indicating cells undergoing active proliferation. The red box indicates the region used to quantify the % BrdU incorporation. ***Right Panel***, T7-SF2 significantly increased LNCaP proliferation 1.6-fold as determined by % BrdU incorporation. Bars represent % BrdU incorporation from an individual experiment in biological triplicate and are representative of multiple experiments.

B) Growth curves, as determined by trypan blue exclusion, comparing stable LNCaP cells transduced with: lentiviral control (CON, Black line) or T7-tagged SF2 (T7-SF2, Red line). Cell numbers are from three independent experiments each expressed relative to Day 0, significant difference at Day 4.

SF2 expression in LNCaP cells impacts splicing

These data were the central focus of an RO1 proposal (described below in the reportable outcomes section) designed to uncover prostate-specific splicing events. A number of candidate genes were screened based on overlap between SF2: binding events [based on CLIP-seq (10)] or gene expression changes [based on RNAi (11)] in HeLa cells and identified prostate spliced genes using publicly available exon microarrays or RNA-seq data (12-14). As shown **Figure 3**, semi-quantitative PCR identified *ZNF121* as a potential SF2-mediated splicing event in LNCaP cells expressing T7-SF2. ZNF121, is a zinc finger protein that may be involved in transcriptional regulation, and was previously identified as a prostate spliced gene (15). Currently, these preliminary data involving *ZNF121* are being verified. Once verified, further investigation will be performed to identify its potential involvement in SF2-mediated PCa cellular proliferation. Importantly, identification of *ZNF121* splicing, in addition to the previously identified D cyclin splicing, indicates that SF2 mediates alternative splicing in PCa cells.

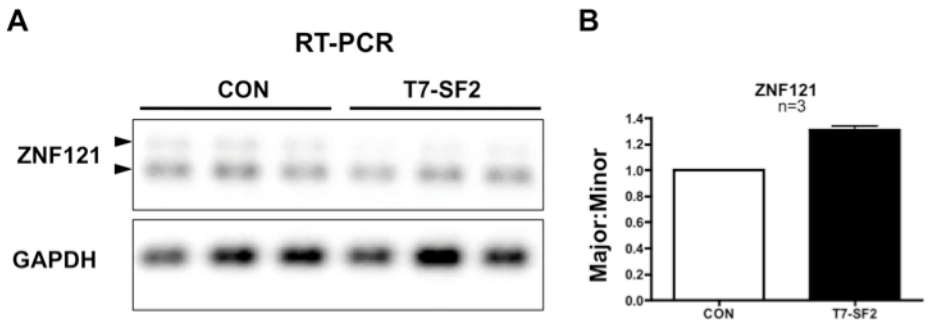

Figure 3. Elevated T7-SF2 alters exon usage of *ZNF121* in LNCaP cells.
A) Representative RT-PCR, for *ZNF121* mRNA (upper) and *GAPDH* mRNA (lower) from stable LNCaP cells transduced with: lentiviral control (CON) or T7-tagged SF2 (T7-SF2). Three biological replicates are shown from one representative experiment. Arrowheads indicate the Major (lower) and Minor (upper) ZNF121 isoforms.
B) Elevated T7-SF2 induced a modest 1.3-fold change in alternative splicing of *ZNF121*. Bars represent the band intensities, as determined using NIH Image gel quantification software, of the Major-to-Minor isoforms relative to control (CON) from three independent experiments.

Subaim 3: Develop RNAi and inhibitor based methods to perform specificity studies.

Summary: To further define the role and ramifications of SF2 in PCa, knockdown studies and inhibitor treatments will be necessary.

Strategy: Obtain constructs and knockdown SF2/*SRSF1* in VCaP cells as these cells express the most SF2 to date (see Completed Accomplishments From 1st Annual Update; Task 1A, Subaim 1) and drugs that alter SF2 functions.

Recent Accomplishments (From 2nd Annual Update):

RNAi-mediated knockdown of SF2 in VCaP cells

These data were the central focus of an RO1 proposal (described below in the reportable outcomes section) designed to complement the over-expression studies as described in Figures 1-3 (above). Attempts to knockdown SF2 in VCaP cells using two different lentiviral shRNA's directed at *SRSF1* mRNA (obtained from an investigator at Temple University) yielded poor knockdown at the protein level and disparate effects between the two constructs (data not shown). Therefore, a pool of siRNA's against *SRSF1* mRNA, purchased from Dharmacon, were transiently transfected into VCaP cells. As shown in **Figure 4**, efficient knockdown of SF2 was achieved as indicated by immunoblot analysis. Furthermore, quantitative PCR determined that *SRSF1* mRNA was reduced approximately 60%. Preliminary, semi-quantitative PCR analysis of alternatively spliced candidate genes (as described for Figure 3) under conditions of SF2 knockdown has identified *ARHGEF11* splicing changes in VCaP cells. *ARHGEF11* was recently identified in a RNA-seq study using prostate tissue (12). *ARHGEF11* is a Rho-guanine nucleotide exchange factor (Rho-GEF) that has been implicated in a diverse number of cellular processes including: glutamate transport, cytoskeleton rearrangements, and epithelial-mesenchymal transition (EMT) (16). Importantly, EMT-like transitions are a characteristic feature of cells undergoing proliferation. In combination with the preliminary data in Figure 3, it is clear that SF2-mediates alternative splicing in PCa cells. It will be important to determine if these splicing events are consistent across multiple PCa cell lines and their involvement in SF2-mediated proliferation (Figure 2).

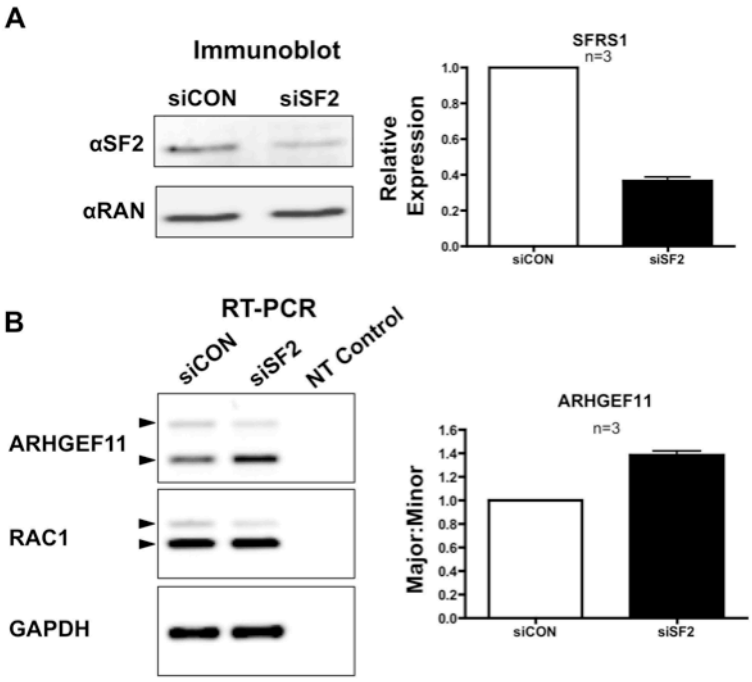

Figure 4. Knockdown of SF2/*SRSF1* alters exon usage of *ARHGEF11* in VCaP cells.
A) ***Left Panel***, Representative immunoblot for endogenous SF2 (upper) and loading control Ran (lower), in VCaP cells, 72hr post-transfection of siRNA control (siCON) or siRNA SF2/*SRSF1* (siSF2) (SMARTpools from Dharmacon). ***Right Panel***, Quantitative PCR of *SRSF1* mRNA indicated a 60% decrease in SF2 mRNA after 72hr of siSF2 treatment in VCaP cell. Relative expression values were determined using the ΔΔCt method relative to GAPDH mRNA. Bars represent three experiments each relative to siCON treatment.
B) ***Left Panel***, Representative RT-PCR, for *ARHGEF11* mRNA (upper), *RAC1* mRNA (middle), and *GAPDH* mRNA (lower) after 72hr of siCON or siSF2 treatment in VCaP cells. Arrowheads indicate the Major (lower) and Minor (upper) *ARHGEF11* and *RAC1* isoforms. *Note*: *RAC1* is a known SF2 splicing target in other tissues but is not affected in VCaP cells based on multiple experiments. ***Right Panel***, Knockdown of SF2 induced a 1.4-fold change in alternative splicing of *ARHGEF11*. Bars represent the band intensities, as determined using NIH Image gel quantification software, of the Major-to-Minor isoforms relative to control (CON) from three independent experiments.

Current Accomplishments:

RNAi-mediated knockdown of SF2 in VCaP cells increases G_2/M arrest

Based on the SF2 overexpression data that indicated a proliferative phenotype (Figure 2). Efforts had been devoted to determining the impact of SF2 knockdown on proliferation in VCaP cells. These data were the central focus of an RO1 proposal (described below in the reportable outcomes section) designed to complement the over-expression studies as described in Figures 1-3 (above). Consistent with a role for SF2 in proliferation of PCa cells, **Figure 5** demonstrates that depletion of SF2 results in a G_2/M arrest.

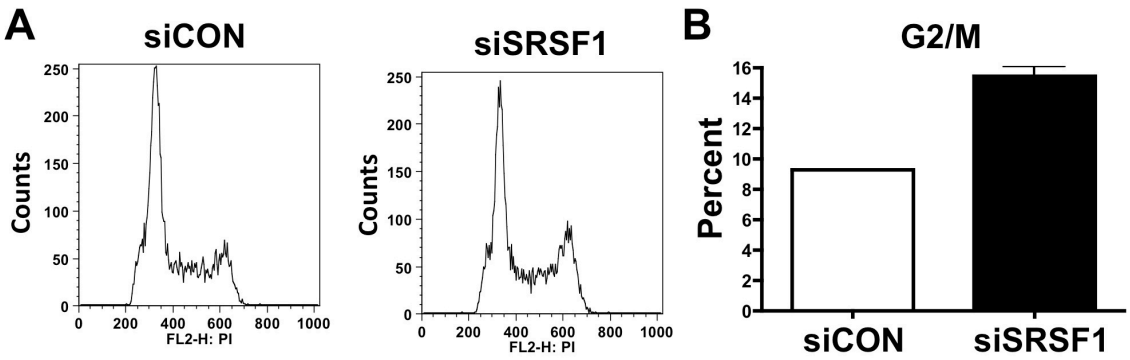

Figure 5. Knockdown of SF2/*SRSF1* increases G_2/M arrest in VCaP cells.
A) Representative cell cycle position using flow cytometry of VCaP cells, 48hr post-transfection of siRNA control (siCON) or siRNA SF2 (siSRSF1) (SMARTpools from Dharmacon). The x-axis denotes cells labeled with propidium iodide (PI), indicating DNA content. The y-axis denotes number of cells counted.
B) FlowJo quantification of data as obtained in Panel A, SF2 knockdown significantly increased G_2/M arrest in VCaP cells. Bars represent percentage of cells in G2/M-phase (n=3).

Aim B: is focused on determining the relevance of SF2-mediated splicing in PCa. Specifically, human PCa specimens with know clinical parameters are to be obtained and archived (Subaim 1). The intent is to stain (Subaim 2) and analyze correlates (Subaim 3 - to be addressed in subsequent years) to determine the relevance to known clinical parameters and/or outcome.

Subaim 1: Obtain and archive clinical prostate specimens.

Summary: Previous analysis was performed using a commercially available PCa tissue microarray (TMA) that had limited clinical information.

Strategy: Obtain human PCa specimens with known clinical parameters to establish the relevance in PCa and correlate with clinical outcome.

Current Accomplishments:

Table 1. Tumors Archived	*Primary Tumors*	*Metastatic Tumors*
Update: 1	3	2
Update: 2	12	2
Update: Final	8	0
Total	**23**	**4**

8 PCa tumors with adjacent non-neoplastic tissue and no metastatic tumors had been archived. The next step would have been to process these tissues collected (**Table 1**) for SF2 and Ki67 to validate the TMA data (Figure 7, below) and proliferation phenotype in cells (Figure 2) and perform correlations as described in Subaims 2 (below) and Future Subaim 3 (described above). In addition, a few prostate tumors with adjacent non-neoplastic tissue were selected for immunoblot analysis of SF2 to validate the immunohistochemical observations (Figure 7, below). As shown in **Figure 6**, PCa tissue from 4 patient specimens had increased SF2 as compared to adjacent non-neoplastic tissue. Li-COR-based image quantification revealed a 3.7-fold increase in SF2 in tumors; thus, confirming the increased SF2 expression by immunohistochemistry (Figure 7, below).

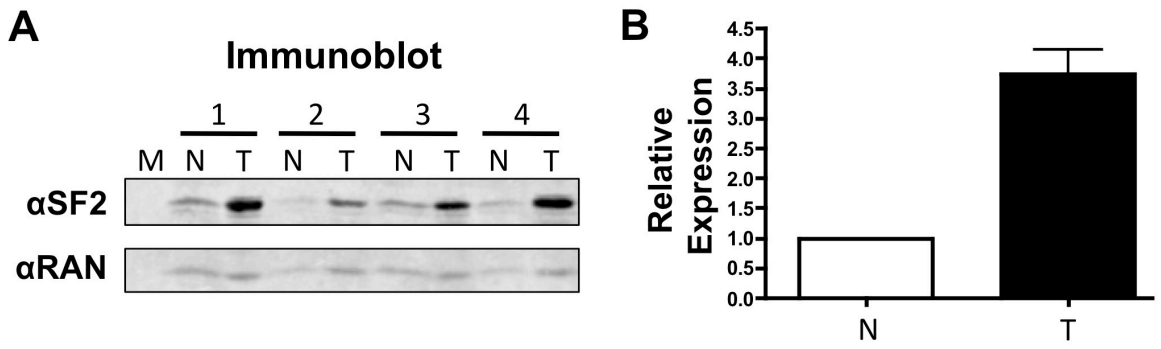

Figure 6. SF2 is increased in PCa tumors.
A) Immunoblot for endogenous SF2 (upper) and RAN loading control (lower), in 4 patient specimens with primary prostate tumors (T) and adjacent non-neoplastic prostate tissue (N). Molecular weight (M) is shown.
B) Li-COR quantification of Panel A. Bars represent SF2 levels relative to RAN. The expression of SF2 in each tumor is adjusted to its appropriate non-neoplastic tissue and is set to 1.

Subaim 2: Immunohistochemical staining of archived human specimens obtained.

Summary: While it is known that the spliced isoform of cyclin D1 is elevated in human PCa as compared to non-neoplastic tissue, and correlates with SF2 levels, as described below. It is unknown what the association of these proteins is to clinical parameters. Thus, assessment of either of these factors in specimens with known clinical parameters and/or outcome is critical for establishing relevance.

Strategy: To accomplish this objective the strategy is relatively straightforward; stain the collected tumors with available antibodies that have been shown to be specific in prostate tissue. However, based on limitations described in more detail below, the AQUA system (as suggested in the initial Proposal) did not provide the desired mechanistic insight or general compatibility. Therefore, focus was on optimizing the published antibodies for a method compatible with other published staining protocols (*i.e.*, Ki67) and more amenable to analyzing larger cohorts of specimens.

Completed Accomplishments (From 1st Annual Update):
Fluorescence-based AQUA analysis using a human PCa TMA identified a significant correlation, with glandular resolution, between SF2 and the spliced isoform of cyclin D1 and these data have been published. However, the AQUA platform was unable to determine correlations at the cellular level using serial-section stained slides and is not very compatible for direct comparisons with most DAB-based staining protocols available. Furthermore, using this small cohort only a trend was discernable between SF2 levels and tumor grade. Thus, previous efforts had been devoted to the development of a cross platform and cost-effective DAB-based immunohistochemical method to screen a larger cohort of PCa specimens to more accurately ascribe relevance with regard to available clinical parameters and/or outcome.

Recent Accomplishments (From 2nd Annual Update):
SF2 is elevated in human PCa specimens
These data were the central focus of an RO1 proposal (described below in the reportable outcomes section) designed to strengthen the clinical relevance. Utilizing the previously developed immunohistochemical approach, the status of SF2 in human PCa tissue was determined from two commercially available TMA's. As shown in **Figure 7**, SF2 (in Cohort 1) was increased 1.4-fold in PCa tumors (n=30) as compared to non-neoplastic prostate tissue (n=44). Moreover, SF2 (in Cohort 2) was increased 1.9-fold in PCa tumors as compared to adjacent non-neoplastic prostate tissue (n=30). However, in both cohorts SF2 did not correlate with tumor progression as had previously been indicated by AQUA analysis. The reason for this is unclear but may relate to differences in immunohistochemical methods and/or the composition of tumors on the various TMA's. Nonetheless, these data indicate that SF2, as predicted by preliminary analyses in the initial proposal, is elevated in human PCa. Moreover,

the fold changes observed in human tumor specimens are consistent with the changes observed *in vitro* (Figure 1A).

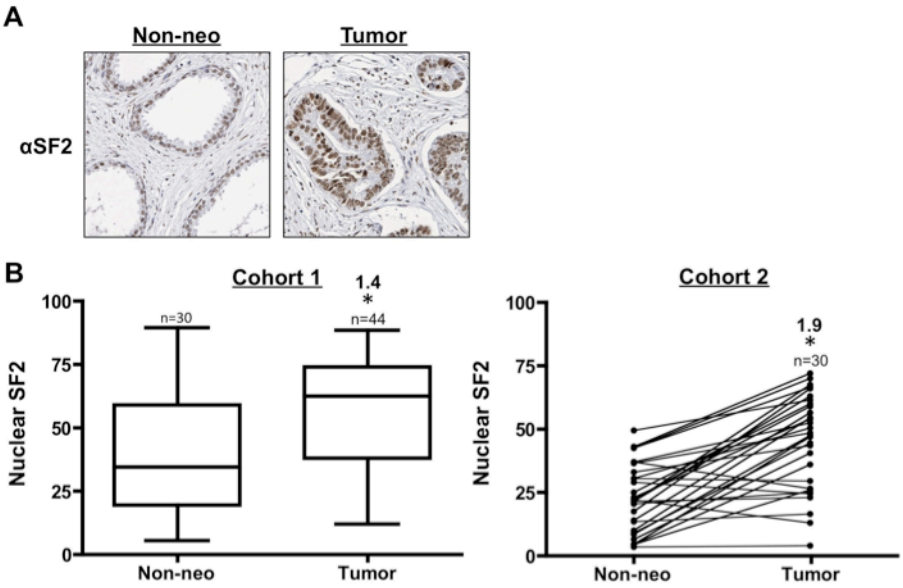

Figure 7. SF2 is elevated in human PCa specimens.
A) Representative immunohistochemical (IHC, 20X magnification) staining for endogenous SF2 (Brown) and hematoxylin counter stain (Blue) in human non-neoplastic prostate tissue (left) and PCa tumor tissue (right) from commercially available tissue microarrays (TMA's). *Note*: SF2 is localized to the nucleus and is primarily in the glandular epithelial cells; however, some stromal and basal cells appear to exhibit nuclear staining consistent with SF2 being an essential splicing factor.
B) ***Left Panel***, Quantification of glandular epithelial SF2 staining in a human tissue microarray (TMA, Cohort 1) indicated a significant 1.4-fold increase in SF2 in PCa tumor tissue (n=44) as compared to non-neoplastic prostate tissue (n=30). Percent nuclear SF2 is represented using a standard box and whisker plot. ***Right Panel***, Quantification of glandular epithelial SF2 staining in a human tissue microarray (TMA, Cohort 2) indicated a significant 1.9-fold increase in SF2 in PCa tumor tissue as compared to matched-adjacent, non-neoplastic prostate tissue (n=30). Percent nuclear SF2 is represented as a before and after plot, each line represents a matched non-neoplastic/tumor pair. *Note*: Both SF2-stained TMA cohorts were scanned and quantified using an AperioScope AT and Spectrum software using the nuclear staining algorithm.

TASK 2

Overview: The general intent of Task 2 is to complement those studies in Task 1 by determination of the *in vivo* consequence of SF2 function and cyclin D1 splicing in the context of PCa development and/or progression (Aim A) and characterization of the response to first line androgen-deprivation therapy (Aim B - to be addressed in subsequent years).

Aim A: is designed to determine the impact of these factors during development/progression of PCa by generating viral constructs (Subaim 1) to genetically modify isolated human or mouse prostate epithelial cell lines (Subaim 2) and perform *in vivo* tissue recombination studies (Subaim 3- to be addressed in subsequent years).

Subaim 1: Generate viral infection constructs.

Summary: Tissue recombination studies, while powerful for mimicking *in vivo* epithelial/stromal interactions are dependent upon the isolation of primary cells or use of transformed but non-tumorigenic cell lines (17). During my training in Owen Witte's laboratory and subsequent communications with Simon Hayward (both experts in the tissue recombination field) it became evident that recombination studies with transfected, prostate-epithelial primary cells or lines is difficult due to the low transfection efficiency. Therefore, it is critical to develop viral constructs to allow production of the desired proteins with high-efficiency.

Strategy: The development of viral constructs is centered on the Gateway cloning method (Invitrogen). The cloning strategy involves initial cloning the gene of interest into a viral entry vector and sequencing. Subsequently, the entry vector is recombined with a viral destination vector that is suitable for expression screening analysis or viral production.

Completed Accomplishments (From 1st Annual Update):
Previous efforts were to generate a lentiviral construct for transduction of T7-SF2 into normal and PCa cells. This construct is completed and has been sequenced. Importantly, this construct was used to generate the LNCaP cell model system with elevated T7-SF2 (Figure 1).

Subaim 2: Isolate/infect normal epithelium to generate genetically modified lines.

Summary: The ability to generate successful tissue recombinants *in vivo* with discernable outcomes requires that a large percentage of epithelial cells contain the desired protein of interest. Thus, the generation of viral constructs, as outlined above in Subaim 1, it is critical. Equally important is the ability to use normal-derived prostate lines and/or isolate primary prostate epithelial cells from mouse or human tissues.

Strategy: Two approaches are currently under consideration to identify epithelial populations amenable to modification for recombination studies. First, recently published data has demonstrated the isolation and characterization of epithelial cells derived from normal and benign human prostate tissue that form tissue recombinants *in vivo*. Second, and somewhat more challenging, is the isolation of embryonic mouse prostate epithelial cells that are traditionally used to form tissue recombinants. Current efforts are underway, with regard to the later approach, to isolate pure populations of mouse epithelial cells.

Completed Accomplishments (From 1st Annual Update):
Using human epithelial cell lines derived from normal and benign prostate tissue were obtained from Simon Hayward (18). Immunoblot analysis for SF2 indicated that the normal cells (NHPrE1) as compared to the benign cells (BHPrE1) express lower amounts of SF2. These cells are as close to human primary isolates and are spontaneously immortalized; thus, eliminating any potential contribution of typical immortalizing factors. As previous efforts to transfect the NHPrE1 cells with T7-SF2 yielded poor efficiency, current efforts are focused on infecting these cells with T7-SF2.

Recent Accomplishments (From 2nd Annual Review):
Primary prostate epithelial cells form prostaspheres
The NHPrE1 epithelial cells are derived from human specimens and should be more equivalent to human primary cells as compared to those derived from mouse. In addition, the established nature of these lines should provide a solid foundation for subsequent modification and more consistent tissue recombinants. Current efforts have been focused on developing prostaspheres that are important for proper tissue recombination (19). As shown in **Figure 8**, using primary adult mouse prostate epithelial cells, a method to form prostaspheres has been achieved. Current efforts will now be focused on forming prostaspheres with the NHPrE1 cells and eventually the NHPrE1 cells expressing T7-SF2.

Current Accomplishments:
Tissue recombination experiments were performed using prostaspheres (described above) that were mixed with freshly isolated mouse embryonic urogential-sinus mesenchmyme (UGM) and implanted under the renal capsules of mice; in order to define the conditions required to form a rudimentary glandular structure that resembles a prostate. Once accomplished, the strategy was to modify epithelial cells with the SF2 viral constructs (described and characterized above) and repeat the tissue recombination experiments to identify if SF2 potentially alters the glandular formation process. Unfortunately, after 15 attempts to generate grafts with glandular structures, I was unable to accomplish this. As shown in **Figure 9**, a representative image of the remaining tissue graft lacking identifiable structures that are characteristic of prostate glands.

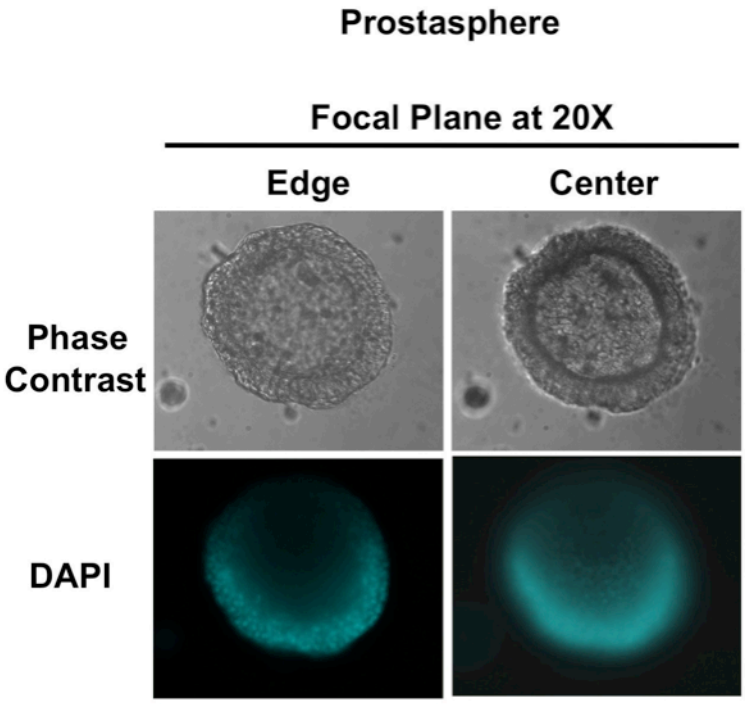

Figure 8. Generation of primary adult mouse prostate epithelial cell prostaspheres.
Epithelial cell organoids "prostaspheres" were generated as described (19). Briefly, primary mouse prostate epithelial cells were isolated from collagenase-digested anterior lobes of adult male mice (8-12weeks). Unsorted epithelial cells (2.5e5/ml) were cultured in Matrigel for up to 5-7 days to allow 3D colonies to form. Formation of prostatspheres were observed around Day 3 and continued to develop reaching a maximal size around Day 7.
Upper Panels, Representative phase contrast image of a prostasphere (Day 7, 20X magnification). Two images of the same prostasphere were taken to demonstrate its 3D nature: one focused on the edge (left) and the other focused on the center (right).
Lower Panels, The prostasphere in the upper panels was stained with nuclear dye (DAPI label, Blue) on Day 7, to demonstrate the approximate number of cells within the sphere. Fluorescence images were taken in the DAPI channel.

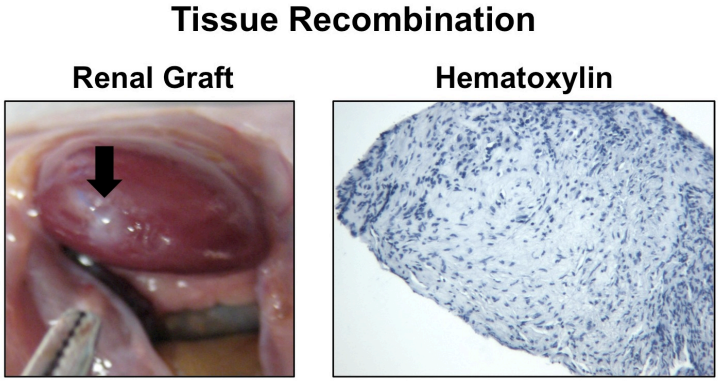

Figure 9. Tissue recombination using prostaspheres and embryonic UGM.
Left Panel) Representative image of a graft [prostaspheres + mouse embryonic urogential-sinus mesenchyme (UGM)] under the renal capsule of a mouse. Arrow indicates graft ~4wks.
Right Panel) The graft in the left panel was processed and stained with hematoxylin (10X image) to evaluate the cells remaining in the graft and the potential for gland-like structures indicative of the formation of a rudimentary prostate. *Note*: no glandular structures were observed in 15 separate grafts.

Based on these data, it was concluded that additional methodologies would be needed to accomplish future goals. Therefore, a collaborative effort (as described in the reportable outcomes section and below) was initiated to develop a more low-cost and higher throughput approach to study SF2 and/or therapeutics that could alter its function. To this end, an *ev vivo* assay was developed that would allow direct assessment of human PCa tissue in the context of its endogenous microenvironment. This *ex vivo* approach is simple, as minced primary tissue (1-3mm^3) is placed on a sterile dental sponge and cultured in standard laboratory cell culture media. In collaboration, this method was very consistent across multiple laboratories (Centenera, **Comstock**, *et.al.*; 2013. *submitted*) and proved to be an effective method to test therapeutics using primary tumor tissue one-step removed from the patient (**Comstock**, *et.al*.; 2013. *Oncogene, in press*). The next step, would have been to evaluate and/or manipulate SF2 and assess proliferation and splicing in primary PCa tissue *ex vivo*.

KEY RESEARCH ACCOMPLISHMENTS

Task 1 - Impact of SF2/cyclin D1 splicing in PCa cells (Aim A) and tumors (Aim B).

Previous:

•Identified multiple representative PCa cell model systems to manipulate SF2.

•Manipulated D-cyclins in the LNCaP cell model system (published (9)) and identified CLK1, a known SF2-associated kinase, as a potential signaling node in PCa.

•Validated that SF2 expression promotes cyclin D1 splicing in PCa cells (published (8)) and characterized a robust and transient over-expression of SF2 results in transcriptional changes in genes associated with SF2.

•Identified a positive correlation (using the AQUA platform) between SF2 and the spliced isoform of cyclin D1 in PCa specimens (published (8)). Due to a small cohort size only a trend was observed for SF2 and Gleason grade; thus, a more cost-effective immunohistochemical method has been developed to screen a larger cohorts of specimens.

Recent:

•Developed stable LNCaP cells that express T7-SF2 to evaluate the role of SF2 in PCa progression (**Figure 1**).

•Identified that T7-SF2 expression increases PCa cellular proliferation and growth (**Figure 2**).

•Assessed potential splicing candidates in LNCaP cells expressing T7-SF2, and identified *ZNF121* as a potential PCa splicing target upon elevated SF2 (**Figure 3**).

•Developed an RNAi approach to knockdown SF2 in VCaP cells, and identified *ARHGEF11* as a potential PCa splicing target upon loss of SF2 (**Figure 4**).

•Characterized SF2 status by IHC and confirmed its elevated status in human PCa specimens (**Figure 7**).

Current:

• Determined that VCaP cells after SF2 knockdown have an increased number of cells arrested in G_2/M (**Figure 5**). These data are consistent with a role for SF2 in PCa proliferation.

• SF2 was observed, by immunoblot, to be increased in a subset of archived human PCa tumors as compared to adjacent non-neoplastic tissue (**Figure 6**). These data validate the findings as determined by immunohistochemistry.

Task 2 - Impact of SF2/cyclin D1 splicing in recombination (Aim A) and therapy (Aim B).

Previous:

•Generated T7-SF2 constructs for lentiviral infection of isolated prostate epithelial cells in order to determine the *in vivo* impact on tumor initiation and splicing.

•Characterized SF2 levels in spontaneously immortalized cell lines derived from normal and benign human prostate tissue to determine the impact in a mouse tissue recombination model of prostate development.

Recent:

•Using primary mouse epithelial cells, developed the prostasphere methodology that is important for the tissue recombination studies (**Figure 8**).

Current:

• Tissue recombination assays did not yield glandular structures as hoped (**Figure 9**). These data suggest that additional optimization is required.

• In collaboration, a simple *ex vivo* assay was developed (Centenera, **Comstock**, *et.al*., below) and therapeutically tested (**Comstock**, *et.al.*, below) that could be used to evaluate SF2 and its activity in prostate tissues in the context of its natural microenvironment.

REPORTABLE OUTCOMES

Manuscripts

Previous:

1. Olshavsky, **Comstock,** *et.al*.; 2010. Identification of ASF/SF2 as a critical, allele-specific effector of the cyclin D1b oncogene. *Cancer Res*. May 15; 70(10):3975-84.
2. **Comstock**, *et.al*.; 2011. Cyclin D1 is a selective modifier of androgen-dependent signaling and androgen receptor function. *J Biol Chem*. Mar 11; 286(10):8117-27.

Recent:

1. **Comstock**, *et.al*.; 2013. Targeting cell cycle and hormone receptor pathways in cancer. *Oncogene*. In Press.

Current:

1. Balasubramaniam, **Comstock,** *et.al*.; 2013. Aberrant BAF57 signaling facilitates pro-metastatic phenotypes. *Clin Cancer Res*. Epub Mar 14. PMID: 23493350.
2. Centenera, **Comstock**, *et.al.*; 2013. Leveraging *ex vivo* culture of solid tumor tissues for molecular research and preclinical drug discovery. *Submitted*.

Abstracts/Presentations/Talks

Previous:

1. **Comstock,** *et. al.*; Keystone Symposia on Nuclear Receptors. Keystone, CO. March 2010. Poster Presentation/Abstract.

Recent:

1. "SF2/ASF in Prostate Cancer". CTCC. Thomas Jefferson University; Philadelphia, PA. April 2012. Invited Talk.
2. "SF2 and Splicing in Prostate Cancer". RNA/Protein (RNP) Discussion Group. University of Pennsylvania; Philadelphia, PA. May 2012. Invited Talk.

Patents: None

Degrees obtained: N/A

Development of cell lines: LNCaP-iLuc/Puro (LNCaP cells infected with luciferase control and stably selected by puromycin) and LNCaP-iT7-SF2/Puro (LNCaP cells infected with T7-tagged SF2 and stably selected by puromycin). Cells have been given to my former postdoctoral advisor Karen E. Knudsen (Thomas Jefferson University) for funding and employment reasons described below.

Informatics: None

Funding: Based on data obtained from this grant, I applied for an RO1 in June 2012. The proposal was reviewed by the Cancer and Molecular Pathobiology (CAMP) Study Section and received a percentile score of 42%. However, in July of 2012 I was given a non-renewal of contract and was unable to revise and resubmit.

Employment/Research opportunities: Due to the comments described above, in the funding section, a large percentage of my remaining time was dedicated to finding gainful employment that would allow me the opportunity to continue my research and support my family. I applied to twelve academic positions at tier two schools (involving primarily teaching and secondarily a research program involving undergraduates). As of the writing of this update, I was unable to secure an interview. In addition, I applied to ~65 research position in industry (ranging from

entry level baccalaureate to senior doctoral scientist) and did not secure an interview. In addition, I also enquired with the Army as to potential health-related research and never heard from the health recruiter. As such, I was unable to continue the project and have given it over to my former postdoctoral advisor as mentioned above.

CONCLUSIONS

Typically, early-stage prostate cancer (PCa) is effectively treated with surgery and/or radiation therapy. However, treatment of more advanced PCa, through traditional modalities that involve androgen deprivation, remains a significant challenge as the disease inevitably transitions into incurable castrate-resistant PCa (CRPC). Therefore, much of the current investigation in the field has centered on the mechanisms that result in CRPC disease. Pertinent to the proposal at hand is a recent development that one CRPC mechanism is alternate splicing of the androgen receptor (AR) that relays androgenic signaling critical for normal prostate function and has been demonstrated to be a key driver of PCa progression. Unfortunately, little information is known about the components, signaling pathways, and mechanisms of splicing in PCa. Previous work has demonstrated that a key regulator (i.e., cyclin D1) of AR signaling and proliferation is alternatively spliced in PCa. Preliminary data, outlined in the original proposal, suggested that a well-known splicing factor (i.e., SF2) regulates cyclin D1 splicing. Importantly, these data have been confirmed and published; wherein, SF2 levels correlated with the spliced form of cyclin D1 and splicing was dependent upon a polymorphism. As outlined in the proposal, it remains to be determined: 1) what is the overall impact and clinical relevance of SF2, cyclin D1, and splicing in PCa cell model systems (Task 1A) and human specimens (Task 1B); 2) what is the consequence of these factors to tumor initiation and progression (Task 2A); and 3) how do these factors contribute to first-line, androgen-deprivation therapy (Task 2B).

Overall Summary: Progress has been made with regard to the Statement of Work and many objectives were completed: ***First***, LNCaP cells expressing stable T7-SF2 have been generated and demonstrate increased cell proliferation, growth, and alternative splicing (Task 1A, Subaim 1/2) and VCaP cells have used to transiently knockdown SF2 and show alternative splicing and G_2/M arrest (Task 1A, Subaim 3). ***Second***, previous preliminary data from our published observations suggests that a potential regulatory loop between D-cyclins and SF2 may exist that should be beneficial for interpretation of outcomes (Tasks 1/2). ***Third***, using the previously developed immunohistochemical protocol SF2 status was shown to be elevated in two independent human PCa tissue cohorts (Task 1B, Subaim 2). However, these data did not confirm a positive trend between SF2 and tumor grade. ***Fourth***, immunoblot analysis confirmed the immunohistochemical data that SF2 is elevated in prostate tumors as compared to adjacent non-neoplastic tissue (Task 1B, Subaim 2). ***Finally***, a prostasphere assay, using primary mouse epithelial cells, has been developed. However, the tissue recombination assays were unsuccessful at generating glandular structures that are indicative of a rudimentary prostate (Task 2). As such, a simple *ex vivo* assay using primary human PCa tissue was developed and characterized therapeutically. Unfortunately, experiments to evaluate and/or manipulate SF2 were not performed due to issues addressed above in the reportable outcomes (funding and employment) section.

So What: Despite one issue (*i.e.*, tissue recombination studies), the current progress is in line with the intended goals of the proposal and, with minimal scientific complications, determined that SF2 is important for PCa cellular proliferation. Future, completion of the outline tasks will provide essential information with regard to splicing, an important and emerging field of prostate biology, and will shed light on the potential ramifications of these factors on first-line therapies.

Alternatives: Three concerns surfaced with regard to completion of the overall objectives of the proposal. ***First***, the collection rate of human PCa tumors with adjacent non-neoplastic tissue or the collection of metastatic tissue has been limited. At current rate, additional sources of tissue had to be indentified in order to define the SF2 status in PCa. According to Table 1, a sufficient number of specimens had been collected to perform a preliminary clinical correlation analysis, which could have been completed if it were not for the funding and employment issues described previously. ***Second***, a number of candidate genes with known splice variants have

been screened and only two potential SF2 targets have been identified (*ZNF121* and *ARHGEF11*). As such, to determine the impact of SF2 on splicing in PCa it is likely that more unbiased approaches may be needed to fully understand the repertoire of SF2-mediated splicing. Third, the tissue recombination studies yielded no prostate structures as intended; thus, making it difficult to continue this line of investigation. However, a simple solution (*i.e.*, *ex vivo* assay) was found that showed excellent promise at addressing the original objective.

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